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Evaluation and Characterization of Genetically Modified Cotton *Gossypium herbaceum* var. Jayadhar for *Helicoverpa armigera* Resistance

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A B S T R A C T

Previously generated genetically modified 22 (J2-J24) cotton transgenic events expresses *Bacillus thuringiensis* (Bt) derived Cry1Ac protein were evaluated for their performance in the field trial during 2014-15. Event J8 (2.23 µg/g) showed the highest accumulation of Cry1Ac protein. The maximum cumulative larval mortality reached up to 76.16 per cent in Bt Jayadhar J2 event, compared to the negative control which stood at absolute zero as expected. The Bt Jayadhar events were further evaluated for the yield parameters, fibre quality and to confirm presence of *cry*1Ac gene providing resistance against *H. armigera.*

Introduction

Cotton (*Gossypium* spp.) is the most important textile fiber crop and the world's second important oilseed crop after soybean. It occupies a predominant position among all the cash crops in India and has retained its unique fame as the "King of Fiber" and the "White Gold" because of its higher economic value among all the cash crops. In 2015-16 recorded productivity was 527.49 kg per lint hectare; however, still lower than the world average of about 701 to 766 kg per hectare and the production was 6.21 million tons against the world's total production of 21.81 million tons. Karnataka is one of the major cotton producing crop which occupies 0.59 million hectares area with the production of 0.41 million tons and 695.06 kg per hectares productivity. (CAB and ICAC Cotton, July, 2016). In Karnataka the Bt cotton growing area was 6 per cent in 2002, and it get consistently increased over a period of time. Cotton crop is infested by more than 160 species of insects of which bollworm infestation results in severe yield loss and majority of the farmers depend upon the use of chemical insecticides. The sucking pest complex comprising of aphids, jassids, thrips, and whitefly are widespread and fairly serious. The bollworm, *Helicoverpa armigera* (Hübner (Lepidoptera: Noctuidae) is one of the most devastating insect pests worldwide, infesting about 300 plant species (Sarate *et al.*, 2012). Due to Bt cotton cultivation there is an improvement in the yield level by reducing the incidence of bollworms. It reduced the use of pesticides resulting in a

reduction of cost of cultivation (Bengi *et al.*, 2015). It also resulted in increase in cotton area and helps in promoting eco-friendly cultivation of cotton resulting in reduction of environmental pollution and health hazard risks which helps in better cotton production.

In Karnataka, a vast tract of dry land and dry farming areas occur starting from Dharwad to Raichur and in this belt *Desi* cotton *Gossypium herbaceum* var. Jayadhar is cultivated. At least 60 per cent of the land that every farmer owned was set aside for growing Jayadhar cotton. In these varieties farmers are never using any insecticides with a notion that they are resistant to insect pests along with moisture stress as they are *Desi* cotton. However, it is not true. *Desi* cotton although known for resistance to sucking pests and moisture stress but are highly susceptible to bollworms. As a result of this, presently the yield level of Jayadhar cotton has come down to 1-2 q/ha as against potential yield 8-10 q/ha.

Therefore, transfer of *Bt* gene like *cry*1Ac may make this variety resistant to bollworms benefiting the farmers in the said cropping systems. Hence, in present study twenty-two most promising transgenic lines (events) generated during 2010-11 were evaluated in both transgenic greenhouse and open field contained condition with the permission of Review Committee on Genetic Manipulation (RCGM) during 2014-15.

Materials and Methods

Experimental site

Field experiment was conducted during *rabi* 2014-15 at University of Agricultural Sciences, Agriculture Research Station, Dharwad. An investigation was carried to evaluate J2-J24 different Jayadhar Bt cotton lines generated as independent transgenic lines referring as event for their performance in the field trial following Randomized Block Design (RBD). Experimental plots consisted of black cotton soil and were homogeneous with respect to nutrient status and leveled by harrowing with a spacing of 90 cm X 20 cm, fertilized with N: P_2O_5: K_2O @ 80:40:40 kg per ha.

Plant material

The plant material consisted of genetically transformed lines of Jayadhar (*G. herbaceum*) cotton containing *cry*1Ac gene. These lines of Jayadhar were developed through shoot apical meristem method of genetic transformation using *Agrobacterium tumefaciens* as vector containing *cry*1Ac gene during the year 2010-11 at University of Agricultural Sciences, Agricultural Research Station, Dharwad Farm.

Gene construct

The disarmed *Agrobacterium* strain EHA-105 harbouring binary vector pBinAR, carrying *cry*1Ac gene cloned between CaMV35S promoter and OCS terminator *II* and *npt II* gene under selectable marker, nopaline synthase (nos) promoter and terminator was obtained from National Research Centre on Plant Biotechnology, IARI, New Delhi was used (Fig. 1).

PCR screening

Genomic DNA was extracted with CTAB method according to Doyle and Doyle (1990) with few modifications. To confirm the presence of a gene in genetically transformed plants of Jayadhar, PCR analysis was carried out using the following pair of gene-specific primers (Fig. 2).

Sigma 1.1 kb Forward -5' CCCAGAAGTTGAAGTACTTGGTGG 3'

Reverse- 5' CCGATATTGAAGGGTCTTCT GTAC 3'

The annealing temperature used for the binding of the forward and reverse primer to the template was 58 ºC. The PCR reaction mixture contained Taq DNA polymerase (New England BioLabs), Taq Buffer (1X) (New England BioLabs), dNTPs (Bangalore Genei), $MgCl_2$ (Bangalore Genei) and *Eppendorf Gradient* PCR (Germany) were used for cyclic amplification of DNA.

Cry protein estimation using ELISA

The quantitative estimation of Cry protein in putative transgenic lines was carried out at 60 days after sowing (DAS) using the Cry1Ac EnviroLogix QuantiPlate ELISA kit as per manufacturer's protocol with few modifications. It was a "sandwich" Enzyme linked Immunosorbent Assay.

Insect bioassay

To perform the insect bio-assay studies, larvae of *Helicoverpa armigera* (Hubner) were reared in the laboratory on artificial diet under controlled laboratory conditions at 26 ± 1 °C, 60–70 per cent relative humidity, and photoperiod of 16:8 h (L/D) in the insect-rearing laboratory at Agricultural Research Station, Dharwad farm.

Fully expanded fourth leaf from second to third nodes below terminal end from 60 DAS transgenic plants were excised and leaf petiole/stalk was inserted in the agar media in the petri plates to maintain moisture in the bioassay petri plates. On each leaf, 10 neonate larvae were released and observations were recorded at different interval (12, 24, 36, 48 and 72 hours). Per cent insect mortality was calculated to obtained bio-efficacy of *cry*1Ac gene in cotton leaves using formula as follows.

Per cent mortality = (number of dead larvae/ number of larvae released) X 100

$$\text{Corrected per cent mortality} = \frac{\text{Treatment mortality} - \text{Control mortality}}{100 - \text{Control mortality}} \times 100$$

With the intention of selecting the higher resistance imparting transgenic event along with the superior yield quality, yield parameters (boll number, boll weight, seed cotton yield per plant, seed index, lint index and ginning out turn) were recorded. Fiber quality of any of the cultivar is also deciding trait towards its adaptability by the farmers. Hence, fiber quality traits such as fiber length, uniformity ratio, fiber fineness, fiber maturity ratio, fiber strength, and fiber elongation were also recorded.

Junction Seq - DNA flanking sequence identification and mapping for GMO events

This experiment carried out using the advanced NGS technologies and bioinformatics tools at SciGenom Alexandria Center for Technology, Turkapally, Hyderabad - 500 078.

Results and Discussion

In several studies it was recorded that minimum quantity of Cry1Ac protein required for killing the insect was 4.1 ng/g (Singh *et al.,* 2015) and according to Kranthi *et al.,* (2005) for durable pest resistance, Bt toxin @ 1.8 µg/g or higher is recommended.

The gene expression can be studied at the transcriptional and translational level using reverse transcriptase PCR (RT-PCR) and enzyme linked immunosorbent assay (ELISA) respectively. A transgenic cotton variety differs in the amount of Cry1Ac expressed throughout the growing season.

Gene expression

Estimation of Cry1Ac protein in the different transgenic progenies was carried out at 60 DAS using Cry1Ac EnviroLogix QuantiPlate ELISA kit. Plant in T_4 generation at 60 DAS showed Cry1Ac protein expression as measured by quantitative ELISA in range of 0.65-2.23 µg/g.

The transgenic event J8 (2.23 µg/g) showed highest accumulation of Cry protein followed by J2 (1.68 µg/g) (Table 1) Cry protein accumulation between events of Jayadhar were varying significantly. All the Bt Jayadhar events showed *cry*1Ac gene expression at the protein level in ELISA. Non-Bt Jayadhar plants were taken as control, which did not show any positive results in ELISA.

Insect bioassay

The effectiveness of gene expression can be effectively understood by actual insect feeding studies through leaf detached bioassay method (Bhaksh *et al.,* 2009). Highest cumulative insect mortality was recorded in event J2 (76.16 %) followed by J12 (63.59 %) and J13 (63.08 %) (Fig. 3; Table 2). The cumulative per cent mortality recorded after 12 hours (14.63 %), 24 hours (24.61 %) and after 36 hours (32.53 %) were significantly differing from each other.

Bhattacharya *et al.,* 2002 showed significant larval mortality ranging from 51.84 to 74.06 per cent and 70-90 per cent (Bhaksh *et al.,* 2009). In previous several studies on checking the efficacy of transgenic events using insect bioassay (Henneberry *et al.,* 2001; Graham *et al.,* 2001; Mandaokar *et al.,* 2000) stated that the highest level of mortality founds at 24 and 48 hours after feeding infestation. It could be because the activity of the Cry1Ac protein is more during this time period.

Evaluation of Bt cotton lines for yield parameters and fibre quality traits

The number of bolls harvested per plant observed in the transgenic lines was found significantly higher than the non-transgenic lines. This strictly indicates that Cry1Ac protein secretion in bolls leads to less infestation of the insects on it. It directly turns into increasing the yield of these lines. Event J2 (13.00) and J8 (12.73) can be considered as best events with respect to the number of bolls harvested (Graph 2). Also, the boll weight of Bt Jayadhar was found to be significantly more than that of non-Bt Jayadhar when compared to overall boll weight (Graph 1). The seed cotton yield/plant obtained was highest in J2 (37.18 g) Bt Jayadhar event compared to the non-Bt Jayadhar (10.71 g) which shows the significant difference in yield while GOT per cent, lint index and seed index was not much different between the Bt and non-Bt Jayadhar (Table 3). This is because only healthy unaffected bolls were collected from both Bt and non-Bt Jayadhar lines. Actually, increase or decrease in yield depends on the yield loss of the non-transgenic counterparts under the same cropping practice (Qaim and Zilberman, 2003). Many reports also support present study that the yield performance of seed cotton in case of Bt genotypes are higher than non-Bt genotypes (Pal *et al.,* 2010; Bennet *et a1*., 2006; Saleem *et al.,* 2010; Devi and Reddy, 2012) because of transgene present in genotypes which offers protection from bollworm attack (Khadi *et al.,* 2008).

Fibre quality characters for Bt Jayadhar events were analyzed using HVI (High Volume Instrument) at Central Institute for Research on Cotton Technology (CIRCOT), regional quality evaluation unit situated at A.R.S., Dharwad farm. It was found that there was not much difference between Bt Jayadhar and non-Bt Jayadhar for fibre quality traits.

Table.1 Quantitative estimation of Cry1Ac protein in Jayadhar Bt cotton events during 2014-2015

S.No.	Events (lines)	Cry1Ac protein in greenhouse grown plants	Cry1Ac protein in field grown plants
1	J2	1.00	1.68
2	J3	0.75	0.90
3	J4	0.78	1.10
4	J5	0.91	1.06
5	J6	0.70	0.85
6	J7	0.65	0.65
7	J8	1.50	2.23
8	J9	0.64	0.91
9	J10	0.80	0.95
10	J11	0.67	0.94
11	J12	0.70	1.28
12	J13	0.68	0.90
13	J14	0.62	0.95
14	J15	1.03	1.11
15	J16	0.74	0.95
16	J17	0.72	0.75
17	J18	0.87	1.01
18	J19	0.85	1.06
19	J20	0.62	0.94
20	J21	0.67	0.90
21	Non –GM	0.05	0.03
	S.Em±	0.04	0.05
	C.D. (P=0.01)	0.14	0.19
	C.V. (%)	13.83	14.25

Table.2 Cumulative corrected per cent mortality of *Helicoverpa armigera* in transgenic events of *G. herbaceaum* var. Jayadhar

Events	12 hours	24 hours	36 hours	48 hours	72 hours
J2	44.12	53.39	64.73	76.16	76.16
J3	6.16	21.83	30.49	47.39	47.39
J5	18.77	32.35	43.92	49.98	60.49
J6	6.16	24.89	32.87	39.98	42.57
J7	27.07	36.75	46.04	49.81	52.22
J8	31.5	43.06	48.17	54.98	54.98
J9	12.29	26.93	41.74	49.98	55.49
J11	0.03	12.64	25.23	22.59	31.89
J12	21.48	27.59	32.87	49.98	63.59
J13	24.19	27.59	35.25	54.81	63.08
J14	6.16	6.16	6.50	21.90	28.79
J15	0.03	8.87	24.73	29.48	32.06
J16	0.03	12.64	21.12	34.48	42.40
J18	21.48	34.53	34.35	39.98	54.98
Non-GM	0.00	0.00	0.00	0.00	0.00
Mean	14.63	24.61	32.53	41.43	47.07
		Fact-1 (events)	Fact-2 (hours)	Interaction (1*2)	
S.Em±		1.27	2.2	4.93	
C.D. @ 0.01		3.52	6.09	13.66	
C. V. (%)		2.74			

Table.3 *Per se* performance of lines of Jayadhar cotton carrying *cry*1Ac gene

Events	Boll weight (g)	Bolls/plant	Seed cotton yield/plant (g)	GOT (%)	Lint index	Seed index
J2	3.26	13.00	37.18	32.67	2.99	6.17
J3	2.50	11.26	24.45	29.58	2.03	4.83
J4	2.64	11.53	28.18	28.52	1.99	5.00
J5	2.56	12.26	29.53	28.32	1.84	4.67
J6	2.50	10.00	23.33	30.07	1.94	4.50
J7	2.50	12.66	29.60	27.81	1.99	5.17
J8	3.00	12.73	36.14	31.42	2.37	5.17
J9	2.73	11.73	30.10	30.49	2.27	5.17
J10	2.80	12.00	31.87	30.39	1.97	4.50
J11	2.70	8.46	21.55	29.59	2.10	5.00
J12	2.83	12.06	31.94	29.99	2.21	5.17
J13	2.56	12.40	29.51	28.52	1.93	4.83
J14	2.66	8.26	20.57	29.58	2.17	5.17
J15	2.63	7.80	19.24	28.65	2.01	5.00
J16	2.76	9.60	25.12	29.64	2.04	4.83
J17	2.83	7.33	19.57	29.69	1.83	4.33
J18	2.73	10.80	28.00	29.47	1.88	4.50
J19	2.76	8.86	23.05	29.33	2.21	5.33
J20	2.60	8.20	19.97	27.67	1.91	5.00
J21	2.80	7.66	20.33	28.23	1.97	5.00
J22	2.63	8.00	19.92	28.73	2.22	5.50
J24	2.70	7.4	18.82	29.01	2.18	5.33
Non-GM	2.50	4.86	10.71	28.52	1.70	4.67
S.Em ±	0.05	0.16	0.94	0.42	0.05	0.12
C.D. (P=0.01)	0.16	0.47	2.83	1.26	0.15	0.35
C. V. (%)	3.46	2.80	6.49	2.48	4.09	4.03

Fig.1 Construct map of the binary vector pBinBt3

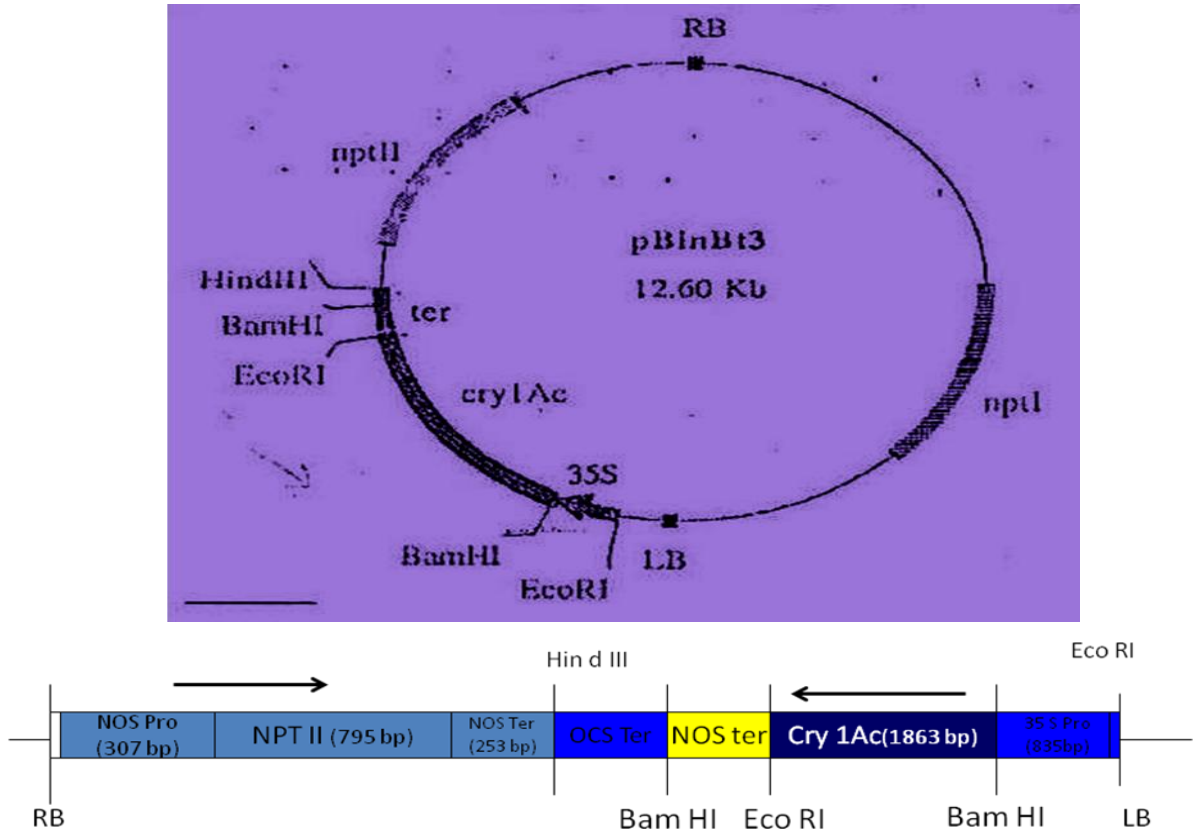
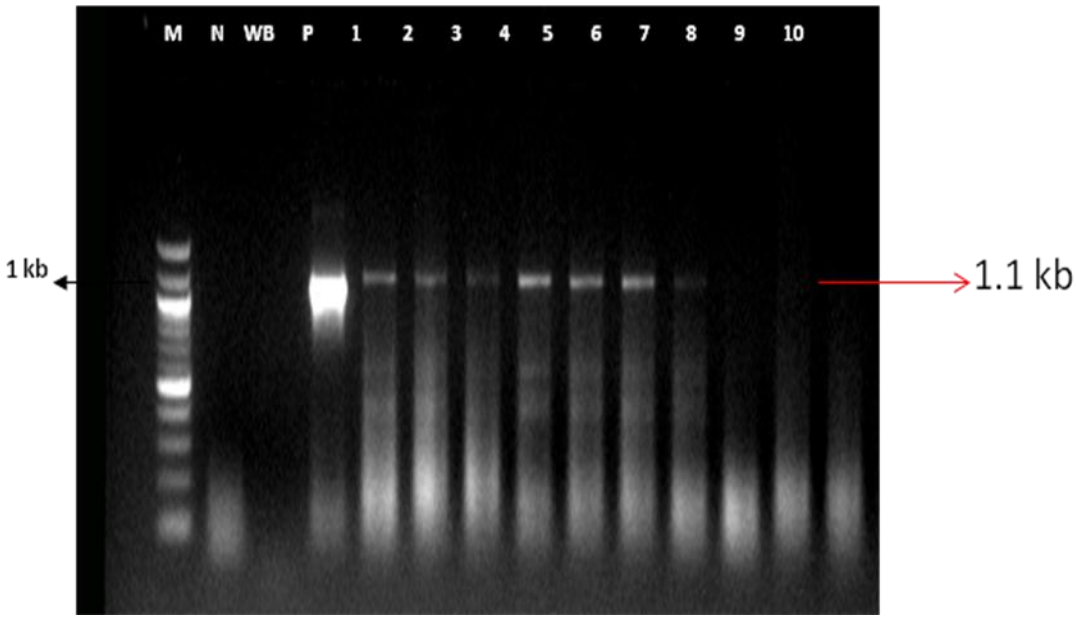


Fig.2 Confirmation of *cry*1Ac transgenic plants through PCR amplification



M: 1 kb ladder, N: Untransformed control control, WB: Reaction mixture without template DNA, P: Plasmid, Lane 1-10: transgenic plants carrying *cry*1Ac gene amplification in transgenic plants.

Fig.3 Leaf feeding insect bioassay of Bt and non-Bt Jayadhar

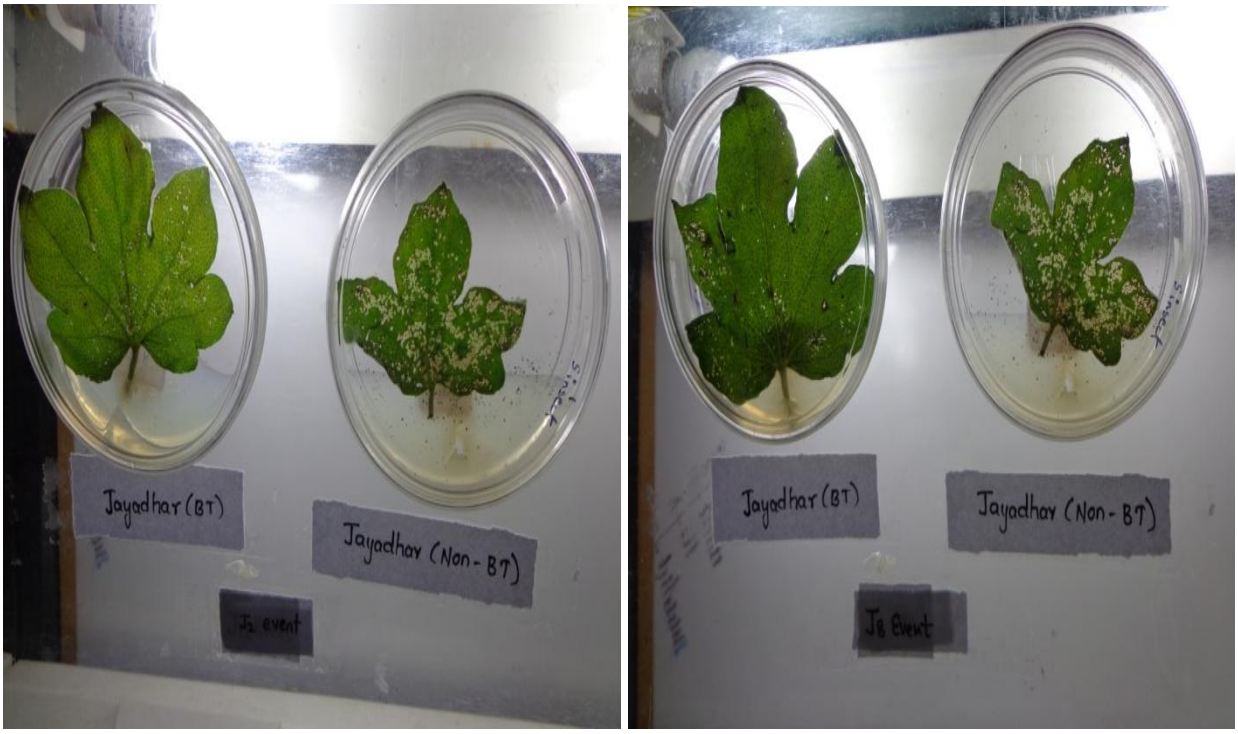
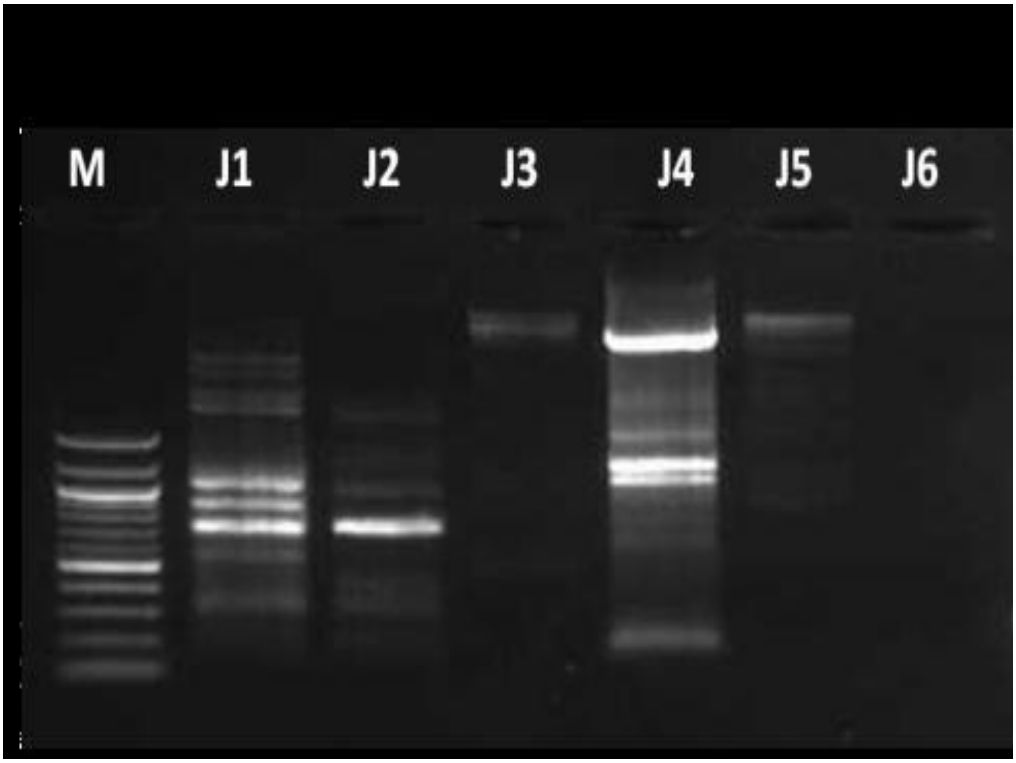
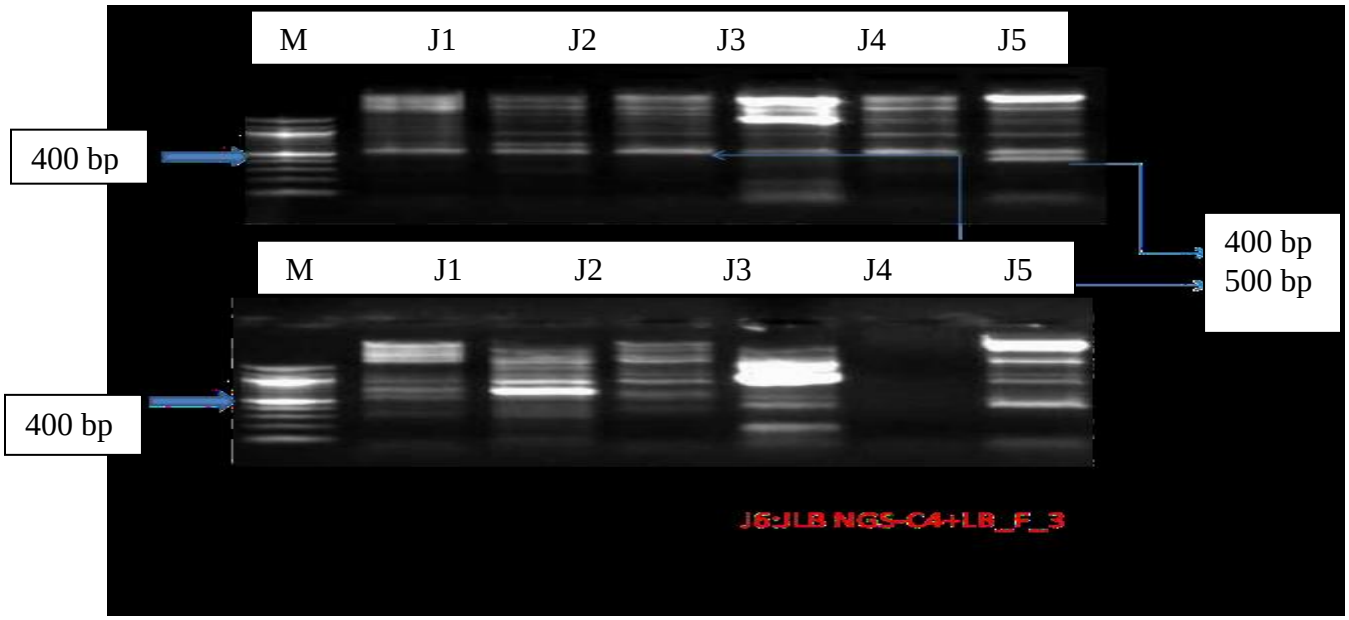


Fig.4 Jayadhar transgenic events confirmation with single primer



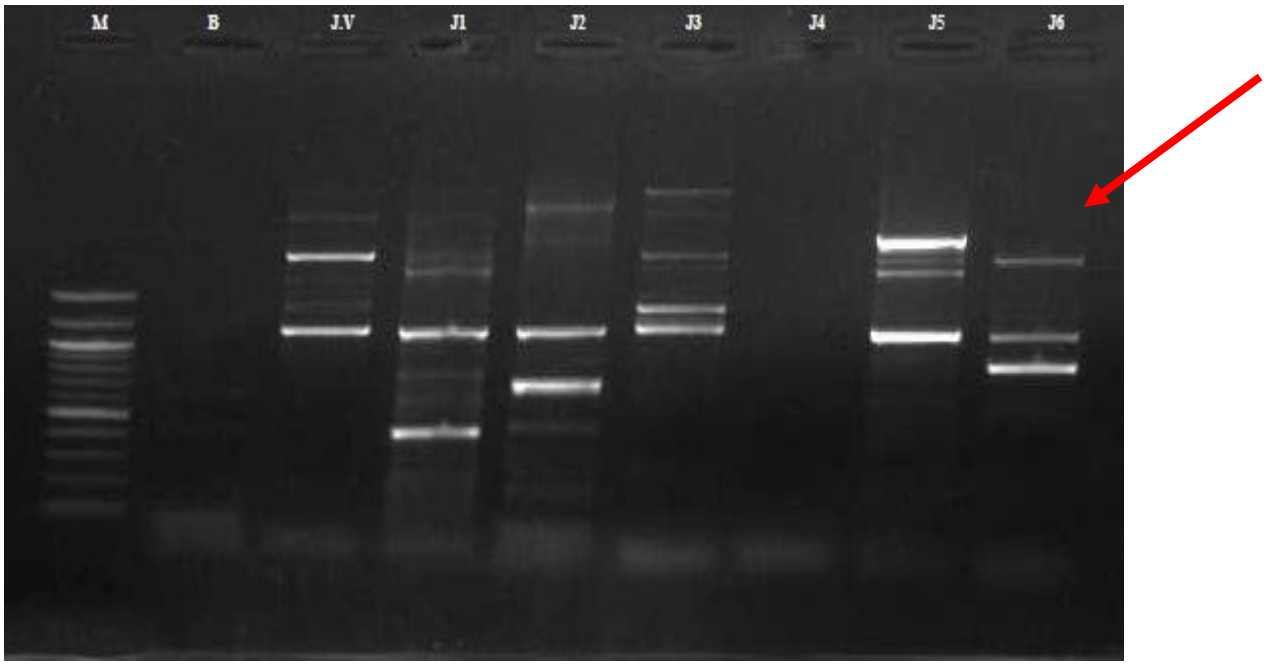
M: Marker, J1: JAY DNA+LB J1 NGS: R1C1, J2: JAY DNA+LB J1 NGS: R1C2, J3: JAY DNA+LBJ1 NGS-C1, J4: JAY DNA+LB J1: NGS-C2, J5: JAY DNA+LB J1 NGS-C3, J6: JAY DNA+LB J1 NGS-C4

Fig.5a First combination PCR: Jaydhar Left Border with LB_F_3 and confirmation primers and control PCR with same combinations of primer



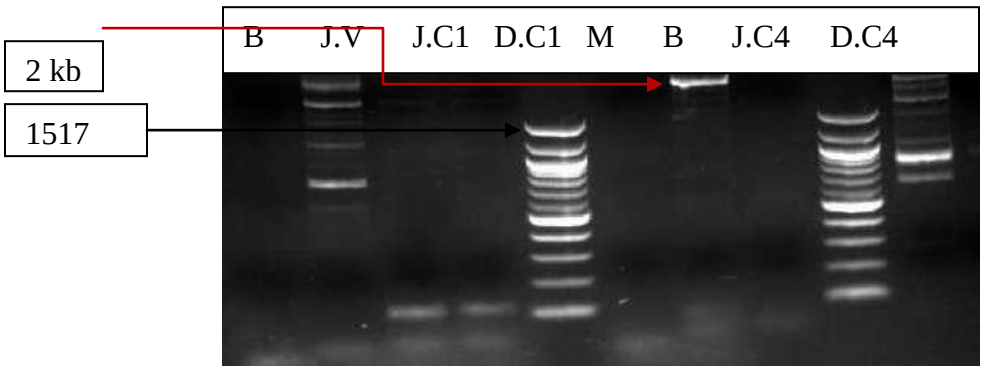
J1: JLB NGS R1C1+ LB_F_3, J2: JLB NGS R2C2+LB_F_3, J3: JLB NGS –C1+LB_F_3, J4: JLB NGS-C2_LB_F_3, J5: JLB NGS-C3_LB_F_3, J6: JLB NGS-C4+LB_F_3

Fig.5b Second combination PCR: Jaydhar Left Border with pBin19 LB F2 with Jaydhar LB confirmation primers



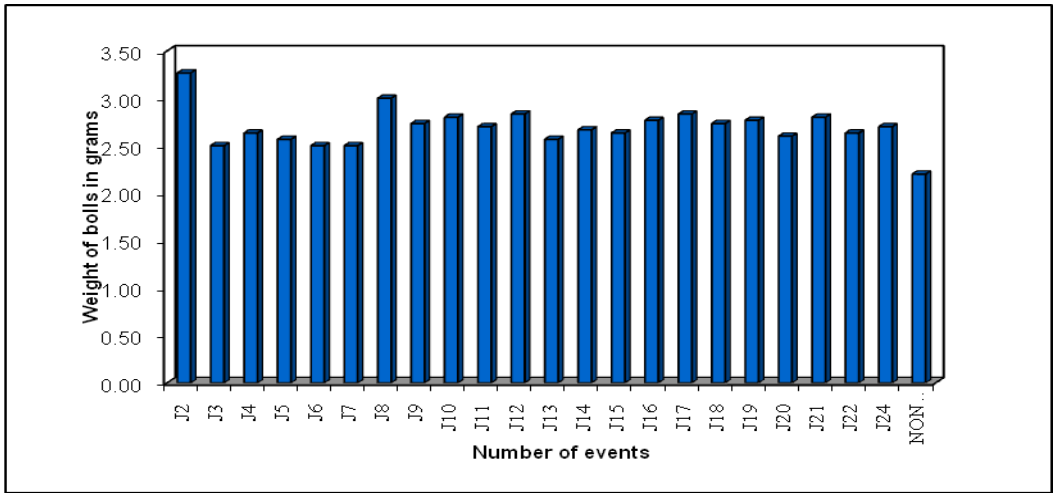
B: Blank, J.V: Jaydhar DNA with vector primer alone. J1: PBIN19 F2 + J1 LB R1C1, J2: PBIN19 F2 + J1 LB R2C2, J3: PBIN 19 F2+ J1 LB C1, J4: PBIN19 F2 + J1 LC C2, J5: PBIN19 F2 + J1 LB C3, J6: PBIN19 F2 + J1 LB C4

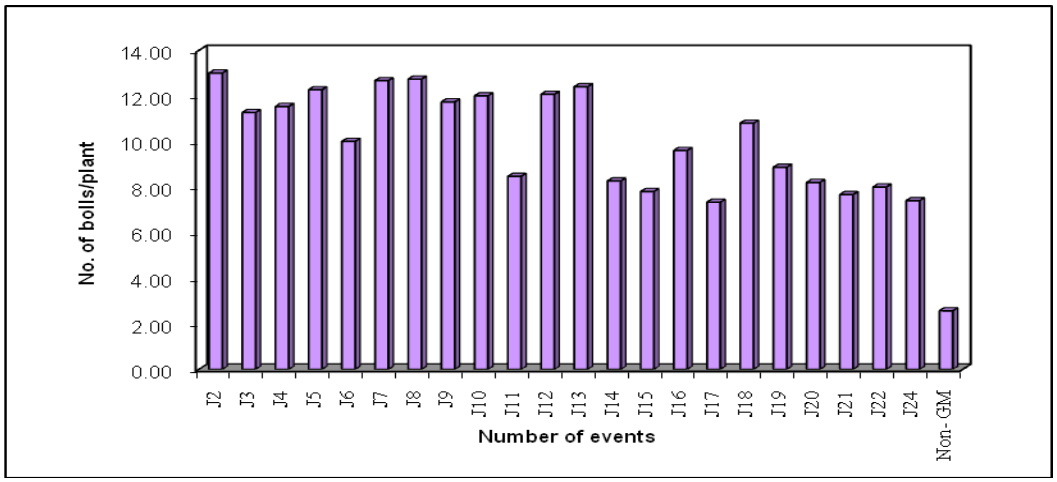
Fig.5c Third combination PCR: Jaydhar Left Border with gene specific and c1,c4 confirmation primers



J.V: Jaydhar DNA + p Bin 19 LB F1 alone, J.C1: Jaydhar DNA+ C1 +pBin19 LBF1, D.C1: DLSA DNA+C1+pBin 19LB F1, B-Blank, J.C4: Jaydhar DNA+ C4+pBin19 LBF1, D.C1: DLSA DNA+C4+ pBin 19LB F1, J1F: Jaydhar DNA+C1+Cry1Ac6F

Graph 1 and 2 Yield parameters (weigh of bolls and No. of bolls/plt) for Bt and non-Bt Jayadhar events





Junction Seq-DNA flanking sequence identification and mapping for GMO events

The site of insertion of T-DNA/Cry1Ac in the cotton genome was identified by recovering the genomic sequences flanking the left border (LB) of T-DNA by NGS sequencing using the reads obtained from sequencing random primer amplicon library. Primary PCR with GSP1 and random primer produced a smear in PCR product (Fig. 4 and Fig. 5a). The multiple bands were noticed in second combination PCR with nested primer 1 and GSP2 where multiple bands were diluted and primary product was used as a template (Fig. 5b).

However there was a reduction in number of multiple bands produced with development of third combination PCR with GSP3 and nested primer 2 (Fig. 5c).

The bright band with approx. 1kb amplicon size were eluted, cloned and sequenced for further process of refining. The amplicons obtained with the left border genomic primer was given for Sanger sequencing for confirmation of sequences.

As no reliable PCR bands were found with left or right border primer combinations we could not proceed further. However, the PCR based methods are limited by quality of DNA. In plants, the final DNA obtained from an extraction has many inhibitors like polysaccharides and polyphenolics that inhibit various enzymatic reactions. When the yield of PCR products are low due to the result of these inhibitions, Sanger sequencing can fail.

The Bt Jayadhar events J2 and J8 are promising based on insect bioassay, protein expression, and yield parameters. Large-scale verification of these two events in future may be required for commercialization.

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